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Full Length Research Paper

High frequency callus induction and plantlet regeneration from different explants of *Picrorhiza kurroa* – *a* medicinal herb of Himalayas

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Picrorhiza kurroa **is a medicinal herb prevalent in the North-Western Himalayan region at an altitude of 3000 - 4300 m. It is a rich source of hepatoprotective picrosides; picroside-i and picroside-ii and other medicinal metabolites such as picroside-iii, picroside-iv, apocynin, androsin, catechol, kutkoside, etc. Being pharmacologically important and listed as an endangered herb, optimization of *in vitro* conditions for callusing and regeneration is of paramount importance not only for the selection of cell lines with enhanced content of phytopharmaceuticals or in the genetic transformation of *P. kurroa*. Moreover, the regeneration hold a great promise in the production of metabolites in cell cultures. Callus cultures were established from different explants such as leaf discs, nodal and root segments of *P. kurroa*. Callus induction was highest (70%) in root segments followed by leaf discs (56.3%) and nodal segments (38.3%) on MS medium supplemented with 2,4-D (2 mg/l) + IBA (0.5 mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v). The callus cultures derived from different explants were differentiated into multiple shoots on MS medium containing different concentrations and combinations of BA, KN and IBA. Regeneration was highest in the calli derived from root segments and leaf discs on MS + BA (2 mg/l) + KN (3 mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) with 76.7 and 72.2% calli forming shoot primordia, respectively. Most of the nodal segment derived calli got differentiated into roots rather shoots. Comparative callusing and shoot regeneration from different explants revealed that root segments are the best explant for *in vitro* studies in *P. kurroa*. The rooted plantlets were acclimatized to the external environment through hardening and eventually transferred to pots.**

Key words: Explants, callusing, regeneration, *Picrorhiza kurroa*.

INTRODUCTION

Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medication. In the International Year of Mountains (2002), *Picrorhiza kurroa* was listed as an 'endangered' herb due to reckless collection from its natural habitat.

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Abbreviations: BA, 6-benzyladenine; **IAA**, indole-3-aceticacid; **NAA**, 1-naphthyleneacetic acid; **IBA**, indole-3-butyric acid; **KN**, kinetin; **MS**, Murashige and Skoog (1962) medium.

P. kurroa Royal ex Benth (Family: Scrophulariaceae) is a perennial herb, also known as kutki or karu mainly found in the North-Western Himalayan regions of India at altitudes of 3000 - 4300 m. *P. kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting. The active constituents are obtained from the roots and rhizomes. *P. kurroa* is a rich source of hepatoprotective picrosides; picroside-i and picroside-ii and other metabolites like picroside-iii, picroside-iv, apocynin, androsin, catechol, kutkoside, etc (Weinges et al., 1972; Stuppner and Wagner, 1989). The medicinal importance of *P. kurroa* is due to its pharmacological properties like hepatopro tec-

tive (Chander et al., 1992), antioxidant (particularly in liver) (Ansari et al., 1988), antiallergic and antiasthamatic (Dorch et al., 1991), anticancerous activity particularly in liver (Joy et al., 2000) and immunomodulatory (Gupta et al., 2006).

A commercial formulation named as Picroliv prepared from *P. kurroa* extracts containing picroside 1 and kutkoside was launched as a hepatoprotective drug after clinical testing (Ansari et al., 1991). Picroliv has also been shown to have immunostimulating effect in hamsters and helping to prevent infections (Puri et al., 1992; Gupta et al., 2006).

The identification of genetically superior strains of medicinal and aromatic plants is a high priority. However, there is no report on the identification of high content strains of *P. kurroa* neither from the natural habitat nor induced through *in vitro* cell cultures. Although conditions for micropropagation has been standardized in *P. kurroa* (Lal et al., 1988; Chandra et al., 2006; Sharma and Sharma, 2003), high content strains can be developed through either by induction of variants in callus cultures from different explants or by genetic manipulation of metabolic pathways. Both these approaches require the standardization of *in vitro* conditions for establishment of high frequency callusing and regeneration in *P. kurroa*. The identification of *in vitro* conditions for high frequency callusing and regeneration will also provide suitable starting material for induction of somatic embryogenesis for rapid multiplication and conservation of *P. kurroa*. This study reports high frequency callusing and regeneration from different explants of *P. kurroa*.

MATERIALS AND METHODS

Selection of plant material and establishment of axenic cultures

The *P. kurroa* plants were procured from the Himalayan Forest Research Institute, Panthaghati, Shimla, H. P., India and planted in pots in a polyhouse at the experimental area of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, India. Shoot apices of pot grown plants were surface sterilized in 0.5% Bavistin and 0.1% mercuric chloride followed by 4 - 5 washings in sterile water. The sterile shoot apices were cultured on MS medium supplemented with IBA (2 mg/l) and KN (3 mg/l), which were found suitable for *in vitro* shoot multiplication of *P. kurroa* (Gautam and Chauhan, unpublished; Figure 1a and b).

Selection and culture of explants

Explants such as leaf discs, nodal segments and root segments were taken from *in vitro* grown plantlets of *P. kurroa* and cultured on MS medium supplemented with different concentrations and combinations of 2,4-D, IAA, NAA and IBA with sucrose 3% (w/v). The pH of the media was adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH and finally agar-agar 0.8% (w/v) was added as a gelling agent. The media were autoclaved at 121°C and 15 lb/in^2 pressure for 15 - 20 min. The autoclaved media were kept in the Laminar Air Flow hood for 1 - 2 days before inoculations. The explants were excised aseptically and cultured on above mentioned media. The cultures were incubated at 16 h light /8 h dark cycle at 25 ± 2°C in plant tissue culture chamber. Data were recorded on days to initiation of callus formation, per cent explants forming calli, days to compete callus formation, callus morphology, and calli forming excessive roots. The data were statistically analyzed for test of significance. The cultures were sub-cultured after every 15 - 20 days on callus induction media for 2 months so as to obtain good growth.

Plantlet regeneration

After the explants were completely transformed into callus mass, the calli or parts thereof were transferred onto regeneration media consisting of MS salts supplemented with different concentrations of BA and KN for differentiating into shoots. Calli of cream color originating from different explants were transferred onto regeneration media and incubated in the plant tissue culture chamber maintained at 16 h light/8 h dark photoperiod. Twenty calli from each explant were cultured on each regeneration medium for regeneration. The data were recorded on days to shoot primordia initiation, per cent calli forming shoot primordia, number of primordia/callus and number of shoots/callus. The regeneration frequency was determined by counting the number of calli forming shoot primordia and plantlets. The data were statistically analyzed for test of significance.

Induction of rooting in shoots and hardening of plantlets

Regenerated shoots were excised from the parent cultures and transferred onto half strength MS media supplemented with different concentrations and combinations of IBA, IAA and NAA for root induction. The cultures were incubated under the same culture conditions as mentioned above. Data for days to root initiation, number of roots/shoot, root length and per cent rooting were recorded and analyzed for test of significance. The rooted shoots were gently removed from the culture vessels, washed under running tap water and transferred to pots containing sand: soil: vermiculite (1:1:1) in the greenhouse conditions for acclimatization and hardening.

RESULTS

Establishment of callus cultures

Callus cultures were initiated from different explants such as leaf discs, nodal and root segments of *P. kurroa* on MS salts supplemented with different concentrations of 2,4-D (0.5 - 2 mg/l) and/or IBA (0.5 - 2.0 mg/l) in combinations with IAA (0.5 - 2.0mg/l) and NAA (0.5 - 2.0 mg/l) (Figure 2a, b and c). Callus formation initiated in all explants within 10 -15 days at the cut surfaces in all test media combinations (Figure 2d). All the explants were transformed into complete callus mass within 4 weeks of culture (Figure 2e). Overall, MS medium supplemented with 2,4-D (2 mg/l) + IBA (0.5 mg/l) was found to be the best for callus induction with frequencies of 70, 56.3 and 38.3% from root segments, leaf discs and nodal segments, respectively (Table 1). Differences in callus morphology and appearance were observed among three explants with calli from leaf discs, root segments and

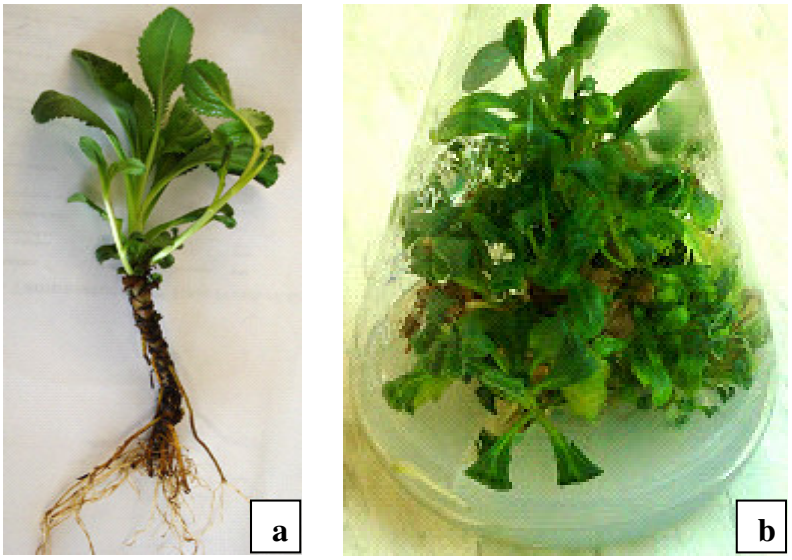

Figure 1. *P. kurroa* plant from pot (a) and shoot apex-derived *in vitro* plantlets (b) as sources of explants.

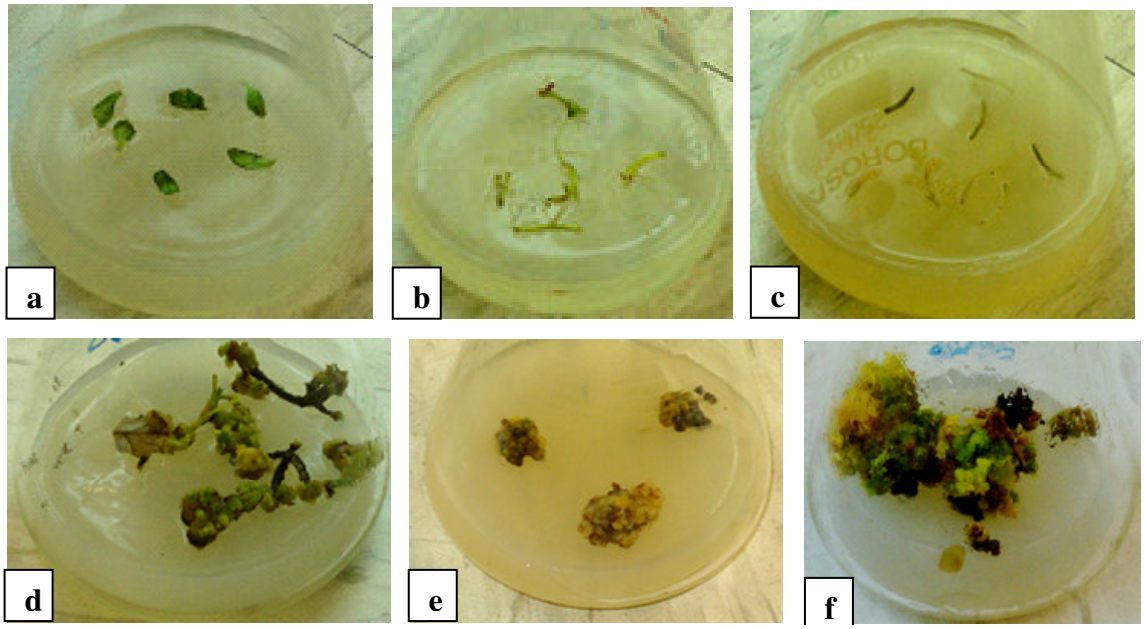

Figure 2. Callus initiation and proliferation from different explants of *P. kurroa*; (a) Leaf segments (b) Nodal segments (c) Root segments (d) Callus initiation (e) Callus growth (f) Callus proliferation.

nodal segments appearing as greenish, creamy white and light brown, respectively. The calli derived from nodal segments turned browner with the passage of time and most of the calli developed into roots even on the callus induction media after subculturing. The conversion of calli into root-like structures was the least in leaf disc-derived calli. Callus cultures derived from different explants were sub-cultured for 6 - 8 weeks so as to proliferate the calli on suitable callus induction media (Figure 2f). During subculturing, some of the calli turned brown and eventually their proliferation stopped. Those calli were not carried further for regeneration. Calli with a creamy appearance were taken for regeneration.

Regeneration of callus cultures into shoots

Proliferating callus cultures or parts thereof were sub-cultured onto regeneration media containing MS salts

Table 1. Effect of MS + auxins on callus induction frequencies from different explants of *P. kurroa*.

MS + Auxins (mg/l)				Explants forming callus (%, mean ± S.E.)*		
2,4-D	IBA	NAA	IAA	Leaf discs	Nodal segments	Root segments
0	0	0	0	0	0	0
0.5	0	0	0	16.3 ± 0.5	12.3 ± 0.5	32.3 ± 0.5
1	0.5	0	0	33.3 ± 0.3	23.6 ± 0.3	60.6 ± 0.5
1.5	0.5	0	0	46.4 ± 0.5	28.2 ± 0.6	66.6 ± 0.5
2	0.5	0	0	56.3 ± 0.3	38.3 ± 0.3	70.0 ± 0.5
2	1	0	0.5	44.5 ± 0.5	36.3 ± 0.3	65.3 ± 0.3
0	1	0.5	1	32.6 ± 0.3	26.6 ± 0.3	43.1 ± 0.6
0	1	1	0	36..3 ± 0.3	17.6 ± 0.3	44.2 ± 0.5
0	2	1.5	0	36.3 ± 0.4	21.6 ± 0.3	56.3 ± 0.3
2	0	1	0.5	38.3 ± 0.6	29.2 ± 0.2	64.7 ± 0.5
2	0	1.5	0.5	39.4 ± 0.5	29.6 ± 0.5	65.6 ± 0.3
2	0	2	0	40.3 ± 0.3	3 2.2± 0.2	66.9 ± 0.5
0	0	1.5	1	44.3 ± 0.3	3 2.4± 0.5	56.5 ± 0.2
0	0	2	1.5	45.6 ± 0.3	35.3 ± 0.3	57.6 ± 0.5
0	0	2	2	46.6 ± 0.4	35.3 ± 0.3	58.3 ± 0.6

*Data represents mean of 20 replicates per treatment in three repeated experiments.

supplemented with different concentrations and combinations of BA, KN and IBA (Table 2). Media containing BA and KN induced shoot primordia formation in the form of green nodular structures. No shoot primordia formation occurred in callus cultures derived from nodal segments on any of the test regeneration media. The MS + BA (2 mg/l) + KN (3 mg/l) was found to be the best for regeneration with 76.7 and 72.2% calli differentiating into shoot primordia from calli derived from root segments and leaf discs, respectively (Figure 3a). The same medium combination was found most suitable for obtaining maximum primordia/callus and the highest number of shoots/callus from calli of both the explants (Figure 3b and c). To avoid browning of callus cultures and for increasing their efficiency of regeneration, the media containing BA (2.0 mg/l) and KN (3.0 mg/l) was supplemented with activated charcoal 0.08% (w/v). The addition of activated charcoal in regeneration media not only reduced the browning of calli, but also enhanced their regeneration frequencies most likely by keeping majority of differentiating cells alive. The shoots regenerated from callus cultures were allowed to grow on regeneration medium for better growth (Figure 3d).

Induction of roots in calli-derived shoots

The shoots formed in the calli derived from leaf and root segments were transferred onto half strength MS media supplemented with different concentrations and combinations of IBA, IAA and NAA for root induction (Table 3, Figure 4a). Data were recorded for days to root initiation, per cent shoots forming roots, number of roots/shoot and root length. Root induction occurred in 9 - 10 days of culturing with highest root induction (70%) on MS medium containing IBA (3 mg/l) followed by 66.6% on MS+ IBA (3 mg/l) + NAA (2 mg/l) (Figure 4b). The same medium was found suitable for more number of roots /shoot and maximum root length/shoot.

Hardening *in vitro* plantlets

Well rooted plantlets were transferred to pots containing autoclaved potting mixture consisting of sand, soil and vermiculite (1:1:1) in the greenhouse for hardening (Figure 4c). Initially, for 10 - 15 days the plantlets were covered with glass beakers to provide sufficient humidity and avoid desiccation till the plantlets showed new growth (Figure 4d). During the hardening process, glass beakers were taken off every day for 1 - 2 h so as to acclimatize the plantlets to external environment (Figure 4e).

DISCUSSION

The overall objective of the current study was to develop an *in vitro* system for establishment of high frequency callusing and regeneration from different explants of *P. kurroa.* There are reports in medicinal and aromatic plants wherein the metabolites of medicinal importance are biosynthesized and accumulate in different organs such as roots, leaves and shoots (Ramachandra and Ravishankar, 2002). Similarly, the accumulations of medicinal compounds do occur in roots, rhizomes and shoots

Table 2. Effect of various media on shoot regeneration in calli- erived from leaf and root explants of *P. kurroa*.

MS + Growth regulator (mg/l)			Days to shoot primordia initiation		Per cent calli forming shoot primordial		Primordia/callus (no.)		Shoots/callus (no.) (mean ± S.E)*	
BA	KN	IBA	Leaf discs	Root segments	Leaf discs	Root segments	Leaf discs	Root segments	Leaf	Root
0	0	0	0	0	0	0	0	0	0	0
0	1	0.5	15-20	15-18	13 ± 0.6	14 ± 0.6	3.0 ± 0.5	3.0 ± 0.5	3.0 ± 0.5	3.6 ± 0.3
0	1.5	1	15-21	14-18	22.2 ± 0.6	24.9 ± 0.7	3.3 ± 0.5	3.6 ± 0.6	3.3 ± 0.3	4.6 ± 0.3
0	2	1.5	15-20	14-18	34.4 ± 0.6	35.6 ± 0.6	4.0 ± 0.3	4.3 ± 0.7	3.6 ± 0.6	5.0 ± 0.5
0	2	2	16-20	15-20	36.6 ± 0.7	40.5 ± 0.5	4.3 ± 0.3	4.3 ± 0.8	4.0 ± 0.5	5.3 ± 0.3
0	3	2	16-20	15-18	40.5 ± 0.5	42.4 ± 0.3	5.0 ± 0.5	5.2 ± 0.2	4.6 ± 0.3	4.6 ± 0.6
0.5	0	1.5	16-21	15-18	22.2 ± 0.6	29.7 ± 0.6	5.3 ± 0.5	5.3 ± 0.3	4.0 ± 0.0	4.7 ± 0.3
1	0	2	15-22	14-18	46.6 ± 0.7	50.0 ± 0.5	3.3 ± 0.2	4.0 ± 0.5	4.3 ± 0.3	4.7 ± 0.3
1.5	0	2	15-22	14-18	51.2 ± 0.2	53.7 ± 0.6	4.3 ± 0.4	4.3 ± 0.4	4.6 ± 0.5	5.3 ± 0.4
2	0	2	15-20	14-17	59.4 ± 0.4	64.2 ± 0.4	4.6 ± 0.5	4.6 ± 0.5	6.3 ± 0.3	5.0 ± 0.5
1	2	0	11-17	10-15	64.2 ± 0.4	64.8 ± 0.5	5.3 ± 0.6	5.6 ± 0.6	6.3 ± 0.3	6.0 ± 0.6
1.5	2.5	0	11-18	10-14	66.4 ± 0.6	70.5 ± 0.5	5.6 ± 0.7	5.6 ± 0.6	6.6 ± 0.5	6.3 ± 0.3
2	3	0	11-18	10-14	72.2 ± 0.6	76.7 ± 0.9	6.3 ± 0.6	6.0 ± 0.5	6.2 ± 0.2	6.6 ± 0.4
1	2	1	11-17	10-15	62.4 ± 0.4	64.2 ± 0.2	5.3 ± 0.3	5.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3
2	3	1.5	11-17	10-15	63.0 ± 0.3	64.8 ± 0.6	5.3 ± 0.3	5.4 ± 0.6	6.3 ± 0.3	6.3 ± 0.3

*Data represents mean of 20 replicates per treatment in three repeated experiments.

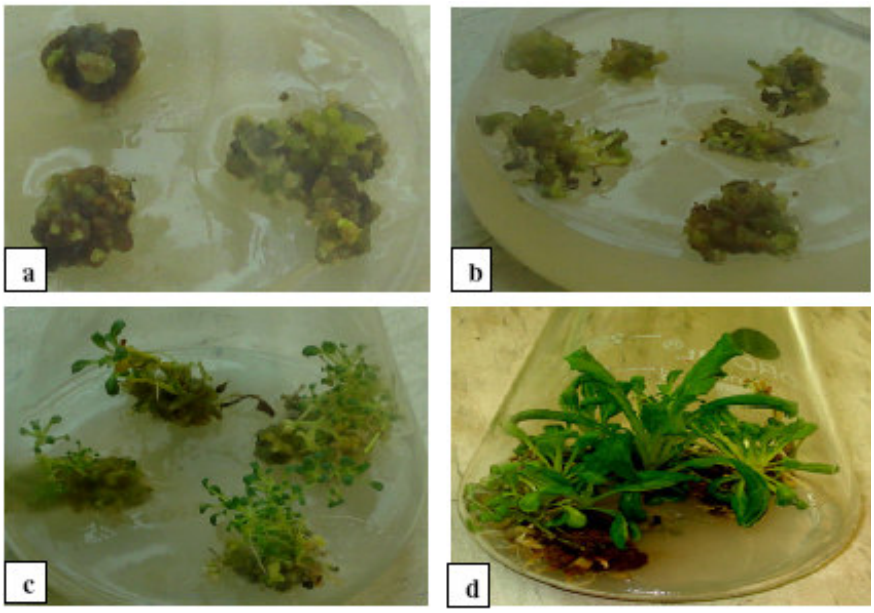

Figure 3. Regeneration of callus cultures derived from leaf and root segments into shoots; (a) Emergence of shoot primordia (b) Differentiating primordia (c) Shoot regeneration (d) Shoot elongation and growth.

of *P. kurroa* (Ansari et al., 1988). The *P. kurroa* has been declared as an endangered medicinal herb due to its heavy exploitation from the natural habitat by various pharmaceutical industries and other vendors dealing with the marketing of medicinal plants. The endangered status thus warrants that tissue culture conditions need to be standardized from different parts of the plant so that the cultures can be used for the production of metabolites under laboratory conditions as has been reported in various medicinal plants so as to relieve pressure from its natural habitat (Vanisree et al., 2004).

The nutrient media forms the main component for

Table 3. Effect of auxins on root induction in *in vitro* regenerated shoots of *P. kurroa*.

1/2 MS + Auxins (mg/l)			Per cent shoots forming roots*	Roots /shoot*	Root length (cm)*	Days to rooting
IBA	IAA	NAA				
0	0	0	0	0	0	0
1	0	0	50.1 ± 0.5	3.6 ± 0.3	3.9 ± 0.2	11-12
2	0	0	61.2 ± 0.3	3.7 ± 0.2	5.4 ± 0.3	12-15
3	0	0	70.1 ± 0.5	3.6 ± 0.5	6.9 ± 0.6	8-10
0	0.5	1	43.3 ± 0.3	5.3 ± 0.6	5.9 ± 0.3	11-12
0	1	2	51.1 ± 0.6	3.6 ± 0.3	5.7 ± 0.4	11-12
2	0	1	52.4 ± 0.4	4.1 ± 0.2	6.2 ± 0.3	11-13
3	0	2	66.6 ± 0.6	5.3 ± 0.7	8.1 ± 0.5	9-10
3	1	0	50.1 ± 0.5	4.3 ± 0.3	6.7 ± 0.2	9-10
3	2	0	53.3 ± 0.3	5.0 ± 0.5	6.9 ± 0.2	9-10

*Values are mean ± S.E.

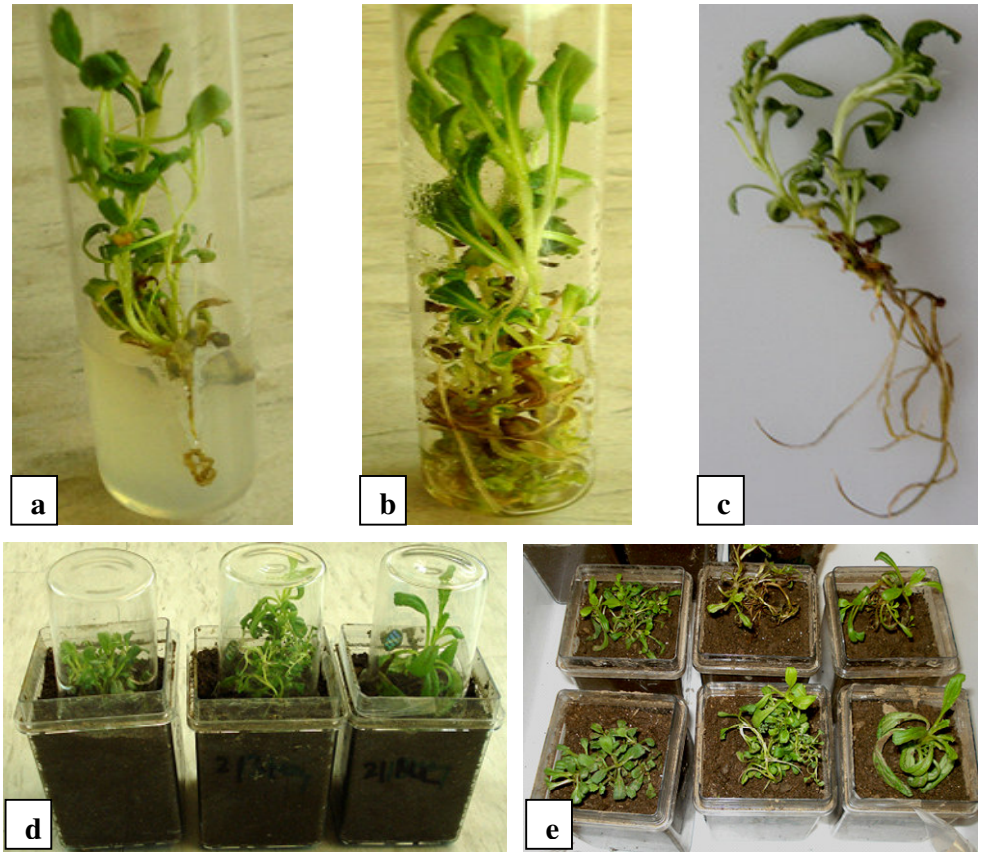

Figure 4. Root induction, hardening and transfer of callus-regenerated plantlets of *P. kurroa*; (a) Shoots in rooting medium (b) Root formation (c) Hardening of plantlets (d) Plantlets in pots.

induction of callusing and plant regeneration in tissue cultures. The major differences in the response of different plant species and different explants to tissue culture conditions lies in the ratio of auxins to cytokinins (Skoog and Miller, 1957). High frequency callusing was achieved from root explants as compared to leaf disc and nodal segments in *P. kurroa* by using different modifications of MS media. Best callus formation was achieved in root explants compared to leaf disc and nodal segment. Similar response of higher callus induction from leaf explants has been reported in other plant species such as *Cichorium intybus*, *Clematis gouriana* Roxb. and

Plumbago zeylanica (Nandagopal et al., 2006; Raja et al., 2007; Rout et al., 1999). During the callus formation, some of the calli turned into excessive roots. The root forming calli have been reported in other plant species such as from root explants of wheat (Chauhan and Singh, 1995). The occurrence of high frequency callusing from root segments of *P. kurroa* is highly desirable because most of the phytopharmaceuticals accumulate in roots. Thus the root-derived callus cultures can be readily used for preparing cell suspension cultures for use in large scale production of medicinally important secondary metabolites in bioreactor conditions or even for understanding the biology of metabolites biosynthesis and accumulation in roots of *P. kurroa* as has been reported in other medicinally important plant species (Smetanska, 2008) .

Shoots were regenerated from different explant-derived calli in regeneration media containing different concentrations and combinations of auxins and cytokinins. Highest regeneration of shoots was observed from callus cultures-derived from root segments as has been reported in other plant species (Chang and Hsing, 1980). The differential response of explants to callusing and regeneration may be due to varying concentrations of endogenous levels of auxins and cytokinins in different explants (Rout and Das, 1997; Saxena et al., 1997; Patra et al., 1998; Jiménez et al., 2005). Rooted shoots were further hardened and transferred to fields; hence a complete protocol for high frequency callus induction and plantlet regeneration has been standardized for *P. kurroa*. The protocol is expected to be of immense practical importance in selection and regeneration of cell lines of *P. kurroa* with enhanced content of medicinally important phytopharmaceuticals. The conditions of callusing and regeneration can also be used in genetic transformation of *P. kurroa* for increased phytopharmaceuticals production through metabolic engineering by using *Agrobacterium*-mediated transformation. Being endangered in its natural habitat, the cell/callus cultures can be cryopreserved for its sustainable conservation (Grout, 2007). Moreover, the endangered status of *P. kurroa* also warrant to take up means for the production of phytopharmaceuticals such as production of hairy root cultures (Verma et al., 2007) or elicitation of cell suspension or callus cultures with biotic or abiotic elicitors for enhanced production of phytopharmaceuticals.

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